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ScienceDirect
Developmental Biology 297 (2006) 550–565
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Genomes & Developmental Control

Identification of *cis*-regulatory elements from the *C. elegans* Hox gene *lin-39* required for embryonic expression and for regulation by the transcription factors LIN-1, LIN-31 and LIN-39

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Received for publication 5 April 2006; revised 3 May 2006; accepted 4 May 2006
Available online 19 May 2006

Abstract

Expression of the *Caenorhabditis elegans* Hox gene *lin-39* begins in the embryo and continues in multiple larval cells, including the P cell lineages that generate ventral cord neurons (VCNs) and vulval precursor cells (VPCs). *lin-39* is regulated by several factors and by Wnt and Ras signaling pathways; however, no *cis*-acting sites mediating *lin-39* regulation have been identified. Here, we describe three elements controlling *lin-39* expression: a 338-bp upstream fragment that directs embryonic expression in P5-P8 and their descendants in the larva, a 247-bp intronic region sufficient for VCN expression, and a 1.3-kb upstream *cis*-regulatory module that drives expression in the VPC P6.p in a Ras-dependent manner. Three *trans*-acting factors regulate expression via the 1.3-kb element. A single binding site for the ETS factor LIN-1 mediates repression in VPCs other than P6.p; however, loss of LIN-1 decreases expression in P6.p. Therefore, LIN-1 acts both negatively and positively on *lin-39* in different VPCs. The Forkhead domain protein LIN-31 also acts positively on *lin-39* in P6.p via this module. Finally, LIN-39 itself binds to this element, suggesting that LIN-39 autoregulates its expression in P6.p. Therefore, we have begun to unravel the *cis*-acting sites regulating *lin-39* Hox gene expression and have shown that *lin-39* is a direct target of the Ras pathway acting via LIN-1 and LIN-31.

Keywords: *lin-39*; *lin-1*; *lin-31*; *C. elegans*; Vulva; Hox; Ras; Gene expression; ETS

Introduction

Hox genes encode homeodomain-containing transcriptional regulators that provide regional identity to cells along the anterior–posterior body axis during metazoan development (reviewed in McGinnis and Krumlauf, 1992; Krumlauf, 1994; Kenyon et al., 1997). The precise control of Hox gene expression is essential for proper development, as demonstrated by the homeotic transformations that result from Hox gene misregulation. Hox gene expression is regulated by extracellular signaling pathways, the Trithorax and Polycomb groups of proteins, and by Hox protein autoregulation and cross-regulation (McGinnis and Krumlauf, 1992; Gellon and McGinnis, 1998; Mann and Morata, 2000; Carroll et al., 2001; Francis and Kingston, 2001). A number of *cis*-acting elements mediating Hox gene regulation have been characterized (Carroll et al., 2001). Regulation at post-translational levels also occurs and involves interaction with Hox cofactors, other Hox proteins, and factors that modulate DNA-binding or Hox protein activity (Mann and Affolter, 1998; Mann and Carroll, 2002; Mann and Morata, 2000).

Hox genes are essential during development of the nematode *Caenorhabditis elegans* (Kenyon et al., 1997). *C. elegans* has

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doi:10.1016/j.ydbio.2006.05.008

six Hox genes in a dispersed cluster (Aboobaker and Blaxter, 2003a,b). Three Hox genes, *ceh-13*, *nob-1*, and *php-3*, are required for embryonic development, while three others, *lin-39*, *mab-5*, and *egl-5*, are only required during post-embryonic development (Clark et al., 1993; Wang et al., 1993; Brunschwig et al., 1999; Van Auken et al., 2000). *C. elegans* Hox genes are regulated by Wnt and RTK/Ras signaling pathways, by homologs of Polycomb and Trithorax group proteins, by cross-regulation, and by other transcription factors (Salser et al., 1993; Kenyon et al., 1997; Eisenmann et al., 1998; Jiang and Sternberg, 1998; Maloof and Kenyon, 1998; Maloof et al., 1999; Ch'ng and Kenyon, 1999; Chamberlin and Thomas, 2000; Alper and Kenyon, 2001; Chen and Han, 2001a; Zhang and Emmons, 2001; Zhang et al., 2003; Ross and Zarkower, 2003; Toker et al., 2003). Recently, *cis*-acting elements regulating expression of two Hox genes, *ceh-13* and *egl-5*, have been identified (Streit et al., 2002; Teng et al., 2004).

We have been investigating the role of the Hox gene *lin-39* during development of the *C. elegans* vulva (Eisenmann et al., 1998; Gleason et al., 2002; Koh et al., 2002; Wagmaister et al., 2006). *lin-39* acts twice during vulval development. In the mid-L1 larval stage, the twelve ventral P cells divide to give an anterior neuroblast daughter (Pn.a cell) and a posterior hypodermal daughter (Pn.p cell). Six of the Pn.p cells, P3.p–P8.p, express *lin-39* and become the Vulval Precursor Cells (VPCs), which are competent to adopt vulval cell fates (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1986; Maloof and Kenyon, 1998). Loss of *lin-39* at this time causes the VPCs to fuse with the hypodermal syncytium, like their anterior and posterior cousins (Clark et al., 1993; Wang et al., 1993). Later, during the L3 larval stage, the interaction of Ras, Notch, and Wnt signaling pathways induces three of the VPCs (P5.p-P7.p) to adopt vulval fates and divide to generate the adult vulva (reviewed in Greenwald, 1997; Sternberg, 2005). Loss of *lin-39* activity at this time causes the VPCs to adopt incorrect vulval fates (Clandinin et al., 1997; Maloof and Kenyon, 1998).

Two extracellular signaling pathways regulate *lin-39* expression during vulval development. First, a Wnt pathway acts in the L2 and L3 stages to maintain *lin-39* expression in the VPCs and ensure proper cell fate specification. Loss of Wnt signaling reduces LIN-39 protein levels in some VPCs and these cells adopt incorrect cell fates (Eisenmann et al., 1998), while overactivation of the Wnt pathway causes ectopic vulval induction that is dependent on *lin-39* (Gleason et al., 2002). Second, at the time of vulval induction in the L3 stage, LIN-39 levels rise in P6.p. This LIN-39 accumulation is dependent on Ras signaling and reflects a transcriptional effect on *lin-39* (Maloof and Kenyon, 1998; Wagmaister et al., 2006).

Several transcription factors regulate *lin-39* expression during vulval development. *lin-1* encodes an ETS domain transcription factor acting downstream of Ras signaling in VPC fate specification (Beitel et al., 1995). In *lin-1* mutants, *lin-39* expression is derepressed in VPCs other than P6.p, suggesting that LIN-1 acts to negatively regulate *lin-39* in those cells (Maloof and Kenyon, 1998; Wagmaister et al., 2006). Two models for LIN-1 function have been proposed. Tan et al. proposed that LIN-1 forms a repressive complex with the winged-helix transcription factor LIN-31, and that phosphorylation of LIN-1 and LIN-31 by MAP kinase disrupts this complex, allowing LIN-31 to act as a transcriptional activator (Tan et al., 1998). More recently, it was proposed that in the absence of Ras signaling, sumoylated LIN-1 represses genes required for adoption of induced vulval cell fates via recruitment of a chromatin remodeling complex, and that phosphorylation of LIN-1 by MAP kinase relieves this repression and may convert LIN-1 into a transcriptional activator (Leight et al., 2005). Consistent with the second model, LIN-1 is required positively for the expression of several genes (Howard and Sundaram, 2002; Tiensuu et al., 2005). However, for none of these LIN-1-regulated genes, including *lin-39*, has direct binding of LIN-1 been demonstrated. In addition to LIN-1, *lin-39* expression in the VPCs is directly or indirectly regulated by the zinc-finger transcription factor SEM-4 (Grant et al., 2000), by the novel protein LIN-25 (Wagmaister et al., 2006) and by SynMuv gene products, which encode components of NuRd and Rb transcriptional regulatory complexes (Chen and Han, 2001a,b).

In this work, we sought to identify *cis*-regulatory elements controlling *lin-39* expression. First, we found a 338-bp promoter fragment that directs expression in P cells in the embryo and in their larval descendants (Pn.a and Pn.p cells) and identified three short DNA sequences important for this expression. This fragment may mediate the initiation and maintenance of *lin-39* expression in these cell types. Second, we show that sequences from the first *lin-39* intron direct expression in a subset of ventral cord neurons (VCNs). Third, we found that a 1.3-kb promoter fragment directs expression in P6.p at the time of vulval induction and also drives expression in the sex myoblast (SM) lineage. Expression from this element in P6.p is dependent on Ras pathway function. We identified three *trans*-acting factors, LIN-1, LIN-31 and LIN-39, that bind this 1.3-kb *cis*-regulatory module. Our results indicate that *lin-39* is directly repressed by the ETS factor LIN-1 in the VPCs in the absence of Ras pathway activity, but that *lin-39* expression in P6.p is positively regulated by binding of LIN-1, LIN-31 and LIN-39 to this *lin-39* promoter fragment. Together, these results account for much of the *lin-39* expression pattern, indicate that *lin-39* may autoregulate its expression in at least two cell types, identify *lin-39* as a direct target of LIN-1 and LIN-31, and show that the ETS factor LIN-1 can act both positively and negatively on the same gene.

Materials and methods

Genetic methods and alleles

Methods for culture and genetic manipulation of *C. elegans* were as described (Brenner, 1974). Wild-type animals were variety Bristol, strain N2. Experiments were performed at 20°C unless otherwise indicated. The reference for most genes and alleles used is (Riddle et al., 1997) LGI: *pry-1(mu38)*; LGII: *cwn-1(ok546)* (Zinovyeva and Forrester, 2005), *deIs6* (this work), *lin-31 (n1053)*; LGIII: *pha-1(e2123)*; LGIV: *dpy-20(e1282)*, *egl-20(n585)*, *let-60 (n1046)*, *lin-1(e1777)*, *lin-45(n2018)*, *unc-5(e53)*; LGV: *him-5(e1490)*; LGX:

lin-15(n765ts). *deIs6* was a spontaneous integrant from strain *pha-1(e2123); Ex [pJW5; ajm-1*::*GFP; pha-1(+)]*.

Transcriptional GFP reporter constructs

Reporter constructs were made by standard molecular biology protocols (Ausubel, 1987). Different *lin-39* genomic regions were amplified by PCR using the cosmids F44F12 or R05A13 as templates. Fragments were cloned upstream of the minimal *pes-10* promoter and GFP coding sequences in the reporter plasmid pPD107.94 (Harfe and Fire, 1998) or upstream of the minimal *egl-18* promoter and GFP coding sequences in the reporter plasmid pKK1 (modified from plasmid pKK62; Koh et al., 2002).

Evolutionarily conserved elements and transcription factor binding sites were altered by scrambling or mutating the sequence in the context of the functional fragment. To scramble an element, SOEing PCR (Splicing by Overlap Extension) (Hobert, 2002) was performed using overlapping oligonucleotides in which the target sequence was randomized but the AT/CG percentage was maintained (Natarajan et al., 2004). Sequences of primers used to create deletion constructs and mutated sites are available on request.

Generation of the transgenic lines

Transgenic worms were obtained by standard DNA microinjection techniques (Mello and Fire, 1995). *pha-1(e2123)* and *dpy-20(e1282)* worms were microinjected with GFP reporter constructs (100 ng/μl and 90 ng/μl, respectively), the *ajm-1*::*GFP* plasmid pJS191 (50 ng/μl and 20 ng/μl, respectively) (Mohler et al., 1998) and the *pha-1(+)* plasmid pC1 (50 ng/μl) (Granato et al., 1994), or the *dpy-20(+)* plasmid pMH86 (90 ng/μl) (Sundaram et al., 1996). Transgenic lines were identified by growth at 25°C (*pha-1*) or rescue of the Dpy phenotype (*dpy-20*). For each reporter construct, two or more independent transgenic lines were analyzed. In general, independent lines showed a similar GFP expression pattern, and data from multiple lines were pooled.

Analysis of strains containing reporter constructs

Worms were synchronized by L1 starvation (Wood, 1988), fed, and allowed to develop at 20°C or 25°C. GFP expression at indicated times of development was observed and photographed using Nomarski differential interference contrast optics and fluorescence microscopy on a Zeiss Axioplan 2 with a Nikon DXM 1200 digital camera and Act-1 software. GFP expression in strains carrying pJW5 and *deIs6* was usually quite weak, requiring the camera for observation. Statistical analyses to determine significance of differences in GFP expression between lines used the Fisher Exact Test.

Purification of LIN-31, LIN-1, LIN-39 and CEH-20 proteins

LIN-31

Escherichia coli strain BL21(DE3) was transformed with glutathione-*S*-transferase (GST)-fusion expression vector pGEX-KG (Guan and Dixon, 1991) containing a full-length *lin-31* cDNA. Exponentially growing cultures were induced with 0.2 mM IPTG at 25° and lysed in buffer containing 50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 50 μg/ml lysozyme. Protein extract was dialyzed against PBS and purified on a glutathione-sepharose column (Amersham Biosciences) using an ÄKTA FPLC (Amersham Biosciences). Affinity-purified GST::LIN-31 was dialyzed against LIN-31 binding buffer [75 mM KCl, 20 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10% glycerol]. GST:LIN-31(N68I) protein was made in a similar manner, beginning with a mutated *lin-31*cDNA corresponding to the *ga57* mutation (Miller et al., 2000).

LIN-1

GST:LIN-1(1–278) protein was purified as described (Miley et al., 2004).

LIN-39 and CEH-20

His-tagged LIN-39 and CEH-20 proteins were purified as described (Koh et al., 2002).

Electrophoretic mobility shift assays

Oligonucleotides used for PCR amplification of the pJW5 fragments used in gel shifts, and the oligonucleotides used directly in gel shifts, are listed in Supplemental Table 1.

LIN-31

DNA fragments were amplified by PCR, separated by agarose gel electrophoresis and purified. GST:LIN-31 protein (1.9 μg) or GST:LIN-31 (N68I) protein (0.9 μg) was incubated with 50 fmol ^{32}P-labeled target DNA at 20°C for 1 h in LIN-31 binding buffer with 0.1 mg/ml BSA and 50 μg/ml polydeoxyinosinate-cytidylate. Protein:DNA complexes were resolved on 7% nondenaturing polyacrylamide gels and visualized using a Storm phosphorimager (Molecular Dynamics).

LIN-1

DNA fragments or oligonucleotides containing individual GGA sites were used. DNA fragments were amplified by PCR, separated by agarose gel electrophoresis, purified, and digested with the appropriate restriction enzymes before labeling. Gel electrophoretic mobility assays with GST:LIN-1(1–278) were performed essentially as described (Miley et al., 2004). The *Drosophila* E74 sequence (Miley et al., 2004) was used as a control for quantification of binding. For quantification, binding in each lane was measured using a Phosphorimager, background was subtracted, and the amount was normalized to the E74 signal (100%).

LIN-39/CEH-20

Gel shifts with 6His:LIN-39 and 6His:CEH-20 proteins, alone or in combination, were performed as described (Koh et al., 2002).

Bioinformatic analysis

Phylogenetic comparisons of *C. elegans lin-39* sequences to similar sequences in *Caenorhabditis briggsae* and *Caenorhabditis remanei* were performed using the programs BLAST (www.ncbi.nlm.nih.gov/BLAST/), CLUSTAL W (www.clustalw.genome.ad.jp), SeqComp, Family RelationsII and Cartwheel (Brown et al., 2005) (cartwheel.caltech.edu) and MLAGAN (lagan.stanford.edu). Candidate transcription factors that might bind to *cis*-acting sites were identified using the Transcription Element Search System (cbil.upenn.edu/tess/) and MatInspector (portal1.0.genomatix.de/products/MatInspector/).

Results

Isolation of genomic fragments that recapitulate aspects of lin-39 expression

lin-39 expression begins in mid-embryogenesis in the P3–P8 cells of the mid-body region (Wang et al., 1993; Wagmaister et al., 2006). After these cells divide in the L1 stage, *lin-39* is expressed in the anterior daughters, P3.a—P8.a, and their neuronal descendants, and in the posterior daughters, P3.p—P8.p, the VPCs (Wang et al., 1993; Maloof and Kenyon, 1998; Wagmaister et al., 2006). At the time of vulval induction, LIN-39 protein levels increase in P6.p in a Ras signaling-dependent manner (Maloof and Kenyon, 1998). Both the Ras and Wnt pathways regulate LIN-39 levels at the transcriptional level (Wagmaister et al., 2006). Additional *lin-39*-expressing cells include the progeny of the neuroblasts QR and QL, and the SMs.

To identify *cis*-acting elements in the *lin-39* gene required for expression and for regulation by the Wnt and Ras pathways, we divided the *lin-39* genomic region (24 kb) into ten fragments that were inserted into the enhancerless *pes-10::GFP* and/or *egl-18::GFP* reporter vectors (Harfe and Fire, 1998; Koh et al.,

2002; Natarajan et al., 2004). Four of the transcriptional reporter constructs showed GFP expression that recapitulated some aspect of the *lin-39* expression pattern (Supplemental Fig. 1). pJW3 contains a 3.1-kb fragment located between 7.2 and 10.3 kb upstream of *lin-39* that drives GFP expression in the P cells P5–P8 in the embryo, and their descendants in the larva (Figs. 1A–D). pJW5 contains a 1.3-kb fragment located between 5.1 and 6.4 kb upstream of the *lin-39* ATG that drives GFP expression in P6.p at the time of vulval induction (Figs. 1E–H) and in the SM descendants (Figs. 1I, J). pJW6 contains a 3.4-kb fragment located between 2.0 and 5.4 kb upstream of *lin-39* that directs expression in P5.p and P6.p and the syncytial hypodermis (Fig. 1K and data not shown), however, expression was observed in few animals (<20%) and was not pursued. pJW8 contains the 1.6 kb first *lin-39* intron, which directs GFP expression in a subset of VCNs (Fig. 1L).

A 338-bp fragment directs embryonic expression in the P cells and their hypodermal and neuronal descendants in the larva

GFP expression from pJW3 was first detected in the embryo in a subset of P cells, P5–P8 (Fig. 1A), and continued after

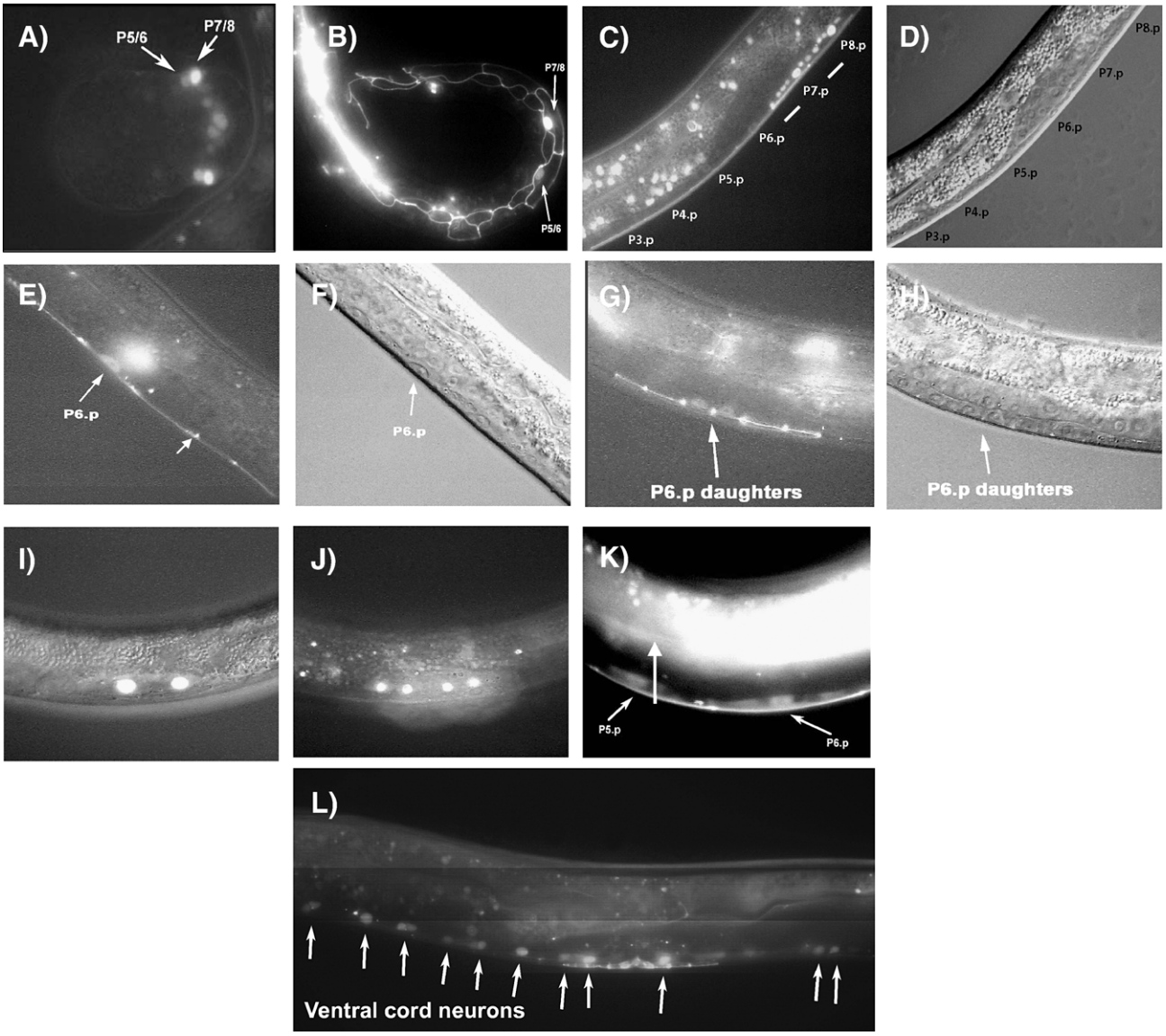


Fig. 1. GFP expression from *lin-39* DNA fragments. Fluorescence and Nomarski images of *pha-1(e2123)* animals with the following constructs as extrachromosomal arrays: (A–D) pJW3; (E–J) pJW5; (K) pJW6; (L) pJW8. In panels (A), (B), (E), (G), (K) and (L), arrows indicate the nuclei of GFP expressing cells. In panels (B), (E), (G), (K) and (L), *ajm-1::GFP* expression shows the junctions of hypodermal cells. Puncta indicate where two cells abut (short arrow in E). Anterior is left, dorsal to the top, except for panel (A). All animals were grown at 25°C. (A) Ventral view of an embryo with expression in P5/6 L/R and P7/8 L/R. (B) early L1 larvae (1 h post-feeding (pf)) with expression in P5/6L and P7/8L. Bright expression is due to *ajm-1::GFP* in the pharynx. (C, D) Early L2 larva (13 h pf) with weak expression in P5–6 descendants and stronger expression in P7–8 descendants. Underline indicates Pn.a-derived VCNs. (E, F) early L3 larva (22 h pf) expressing GFP in P6.p. Bright GFP expression out of the plane of focus is the SM. (G, H) Mid-L3 larva (24 h pf) expressing GFP in the P6.p daughters. (I) Mid-L3 stage larva with expression in two SM descendants. (J) Late L3 stage larvae (26 h pf) with expression in four SM descendants. (K) Early L3 stage larva with expression in P5.p and P6.p. Bright staining is intestine autofluorescence. (L) Late L3 stage larva with expression in VCNs.

hatching (Fig. 1B). After P cell division in the L1 stage, expression was seen in the posterior VPC daughters, P5.p–P8.p, and in VCNs derived from the anterior daughters (Fig. 1C). Expression in the VPCs decreased during the L2–L3 stages and became undetectable by the mid-L4 stage (Table 1). Interestingly, GFP expression from pJW3 forms a gradient, with the strongest and most penetrant GFP expression in P7/8, weaker expression in P5/6, and no detectable expression in P3/4. This expression gradient persists in the descendants of these cells in the larva (Fig. 1C). We previously saw a similar graded expression from a *lin-39*::*GFP* reporter containing the entire *lin-39* upstream region (Wagmaister et al., 2006).

To highlight important *cis*-acting elements in pJW3, we looked for sequences strongly conserved in a similar location upstream of the *lin-39* gene in *C. briggsae*. Four short sequences in pJW3 (13–32 bp; 85%–100% identity) were conserved upstream of *C. briggsae lin-39* (S1–S4; Fig. 2A, and data not shown). The region containing sites S2–S4 was neither necessary nor sufficient for P cell expression, however, a 338-bp fragment, pJW3.9, gave the same pattern of expression as the entire 3.1-kb element in pJW3 (Fig. 2A). A similarly located fragment from upstream of *C. briggsae lin-39*, pJW3.9Cb, directed GFP expression in the same cells as pJW3.9 when injected into *C. elegans* (Fig. 2A and data not shown).

pJW3.9 contains three regions of interest (Fig. 2B): (1) the 23 bp conserved sequence S1, (2) a direct repeat (R1) of the sequence AATTTATC that is partially conserved in *C. briggsae*, and (3) a direct repeat (R2) of the sequence CATTTGTT that is similar to the consensus sequence (CCTTTG(A/T)(A/T)) recognized by Wnt pathway transcription factors of the LEF/TCF family (Riese et al., 1997; Korswagen et al., 2000). Our analysis showed that two of these sequences are needed for expression from pJW3.9. Mutation of the S1 site completely abolished GFP expression in embryos and larva (pJW3.S1m, Fig. 2A), indicating S1 is essential for expression in the P lineages. When the R1 site was deleted or mutated, GFP expression in P7/8 was greatly reduced (pJW3.13 and pJW3.R1m; Fig. 2A; $P < 0.01$). Therefore, direct repeat R1 is necessary but not sufficient (pJW3.11) for robust expression in the descendants of the P cells in the larva. Finally, deletion of 107 bp from pJW3.9 that leaves S1 intact but which deletes sequences including R2, also caused loss of GFP expression (pJW3.11; Figs. 2A, B). However, when the R2 site was mutated, GFP was still expressed (pJW3.R2m; Fig. 2A), indicating that other less conserved sequences in the small region present in pJW3.10 but missing in pJW3.11 must be necessary for expression (Fig. 2B).

In summary, we identified a *cis*-regulatory module that controls embryonic and early larval *lin-39* expression in the P cells and their descendants, including the VPCs. We identified three important sequences in this module: the 23 bp conserved sequence S1, the direct repeat R1, and the 22-bp region next to repeat R2. One or more of these sequences could bind factors that initiate expression of *lin-39* in this lineage in the embryo. Both the R1 site and promoter proximal portion of S1 contain TGATAA sequences predicted to bind GATA-class transcription factors; in addition, the promoter distal portion of S1 contains a sequence (CAATTAGTCA) predicted to bind the AP1 and AP3 factors. However, to date, we have not identified any proteins that act directly via these sites. Expression from pJW3 is not altered in *sem-4* mutants or in mutants in which the Wnt pathway is underactivated or overactivated (data not shown).

Intron 1 directs expression in ventral cord neurons

The 1.6-kb first intron of *lin-39* directs GFP expression in a subset of VCNs (pJW8; Supplemental Fig. 1; Fig. 1K), which began in the early L2 stage and continued throughout larval life (Table 2). We have not determined the specific identity of these neurons, but they are likely to be Pn.a-derived VCNs born in the L1. Interestingly, neuronal expression was observed outside the normal anterior–posterior boundaries of *lin-39* expression (data not shown). There are two sequences (HP1 and HP2) in intron 1 similar to the consensus site for Hox/Pbx heterodimer binding (TGATNNAT(G/T)(G/A) (Mann and Affolter, 1998), which are

Table 1
Expression of pJW3 in the P5–8 lineages during development

Stage [a]	P cell lineage [b]	GFP expression (%) P5.p [c]	GFP expression (%) P6.p [c]	GFP expression (%) P7.p [c]	GFP expression (%) P8.p [c]
Early L1 (2 h)	P cell [d]	36	36	88	88
Mid L1 (7 h)	P cell [d]	53	53	100	100
Late L1 (10 h)	Pn.p cell	47	47	90	90
Early L2 (13 h)	Pn.p cell	24	31	83	83
Mid L2 (16 h)	Pn.p cell	13	17	87	83
Late L2 (18 h)	Pn.p cell	3	17	76	72
Early L3 (21 h)	Pn.p cell	0	0	45	52
Late L3 (24 h)	Pn.px	0	0	37	20
Early L4 (27 h)	Pn.pxxx	0	0	30	30
Mid L4 (31 h)	Pn.pxxx	0	0	0	0

[a] Larval stage and hour post-feeding at 25°C.
[b] Stage of the P cell lineage.
[c] Percentage of animals showing GFP expression in the indicated lineage.
[d] At the P cell stage it is not possible to distinguish P5/P6 and P7/P8, so the numbers are identical. $n = 30$ for all time points.

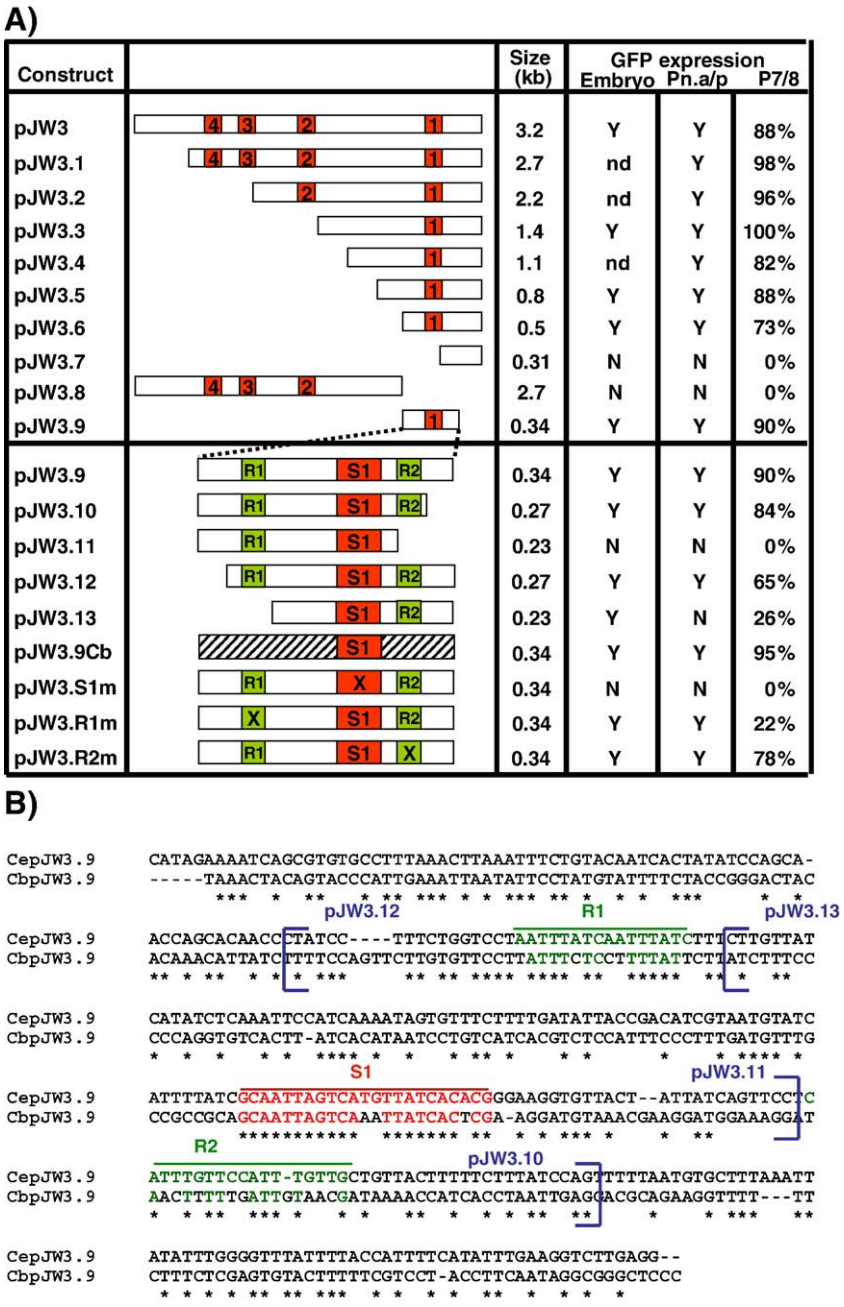


Fig. 2. Deletion analysis of pJW3. Indicated fragments were fused to the *pes-10::GFP* reporter and scored as extrachromosomal arrays. Two or more lines per construct were scored at 25°C with $n > 50$. Red boxes (1–4) indicate sequences conserved between *C. elegans* and *C. briggsae*. Green boxes represent two direct repeats. 'X' indicates mutation of a site. pJW3.9Cb is the corresponding promoter fragment from *C. briggsae lin-39*. 'Embryo' indicates embryonic expression in P5–8 cells; 'Pn.a/p' indicates any expression in P5–8.a and/or P5–8.p in the early L2 stage. 'Y' indicates GFP expression was seen, 'N' indicates no expression was seen. 'P7/8' shows the percentage of early L1 stage (1 h post-feeding) animals with expression in P7/8L or R. 'nd'—result not determined. (B) Sequence of pJW3.9 fragment from *C. elegans* (top) and *C. briggsae* (bottom). Asterisks indicate nucleotide identity. The red overlined sequence is conserved element S1. The green overlined sequences are direct repeats R1 and R2. Blue brackets indicate the left end (pJW3.12, pJW3.13) or right end (pJW3.10, pJW3.11) of four informative deletion derivatives of pJW3.9.

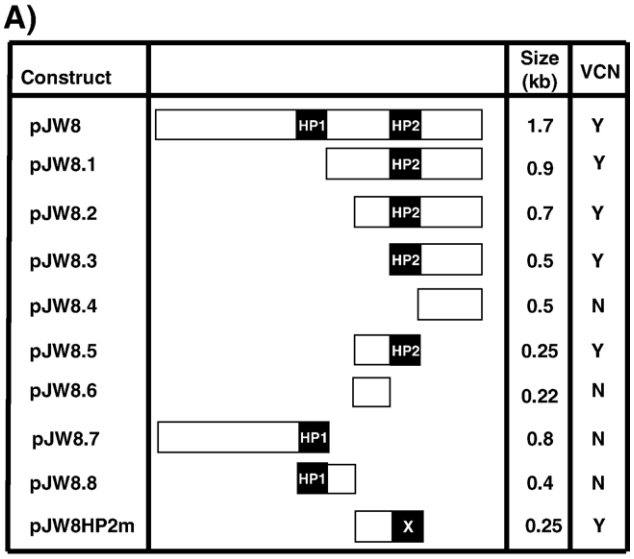
conserved in the first intron of *lin-39* from *C. briggsae* and another closely related nematode, *C. remanei* (Fig. 3). Gel mobility shift assays with purified LIN-39 and its binding partner, the Pbx/Exd homolog CEH-20 (Liu and Fire, 2000), showed that LIN-39 alone bound both sites and bound at a lower concentration in the presence of CEH-20 (data not shown). This result suggests that LIN-39 could autoregulate its own expression in VCNs. Deletion analysis showed that HP1 is neither necessary (pJW8.2) nor sufficient (pJW8.7) for expression in the VCNs, although the number of neurons showing GFP expression was reduced when HP1 was removed (data not shown). We did find a 247-bp fragment containing HP2, pJW8.5, which was necessary and sufficient for VCN expression (Fig. 3A). GFP expression from pJW8.5 was

Table 2
Expression of pJW8 in VCNs during development

Stage [a]	GFP expression (%) VCNs [b]	GFP expression (%) VPCs [c]
Early L1 (1 h)	0	0
Mid L1 (7 h)	0	0
Late L1 (11 h)	0	0
Early L2 (19 h)	15	0
Late L2 (22 h)	77	0
Early L3 (25 h)	68	0
Mid L3 (29 h)	81	4
Late L3 (32 h)	68	0

[a] Larval stage and hour post-feeding at 25°C.
[b] Percentage of animals showing GFP expression in VCNs.
[c] Percentage of animals showing GFP expression in VPCs. $n = 30$ for all time points.

brighter than the original pJW8 construct (data not shown). However, mutation of the HP2 Hox/Pbx site within pJW8.5 (pJW8HP2m) did not disrupt GFP expression, indicating the HP2 LIN-39 binding site is not necessary for VCN expression. Curiously, GFP expression from pJW8HP2m was also now seen in vulval cells, indicating HP2 might mediate repression of *lin-39* in that tissue (data not shown). In summary, a minimal 247-bp sequence from *lin-39* intron 1 drives expression in a subset of VCNs. We did not pursue analysis of this element further.



B)

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C. elegans   HP1    AGATTGAGATTAATGATCGTTCCATCGATT
C. briggsae  HP1    TAATTGAGATTATTGATTGGATGACCCAAA
C. remanei   HP1    CGATTGAGATTAATGACGTGTCATGGAGAG

C. elegans   HP2    AGAGGAAGAGTTTCAAGATTAATTGTTCCCG
C. briggsae  HP2    GAAGGAAGAGTTTCAAGATTAATTGTCTCTT
C. remanei   HP2    GAAGGAAGAGTTTCAAGATTAATTGTCTCTT
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Fig. 3. Deletion analysis of pJW8. (A) Indicated fragments were fused to the *egl-18::GFP* reporter and scored as extrachromosomal arrays. Two or more lines per construct were scored at 25°C with $n > 60$. Dark boxes indicate sequences conserved in *C. elegans* and *C. briggsae* containing Hox/Pbx binding sites (HP1, HP2). 'X' indicates mutation of a site. 'VCN' indicates expression in at least one VCN in late L3 stage to mid L4 stage animals: 'Y' indicates GFP expression was seen, 'N' indicates no expression was seen. (B) HP1 and HP2 sequences conserved between *C. elegans*, *C. briggsae* and *C. remanei*. Underlines indicate putative Hox/Pbx binding sites.

A 1.3-kb lin-39 promoter fragment directs GFP expression in the sex myoblasts and in P6.p at the time of vulval induction

pJW5 contains a 1.3-kb promoter fragment (Supplemental Fig. 1) that drives GFP expression in the P6.p (Figs. 1E–H) and SM lineages (Figs. 1I, J). We detected no GFP expression during embryogenesis or early larval life, but beginning in the L2 stage, we detected GFP expression in both cell types (Table 3). Expression in the SMs and their descendants continued through the L4 stage but disappeared in differentiated vulval and uterine muscles. Expression in P6.p (Fig. 1E) or P6.px cells (Fig. 1G) reached a peak in the mid L3 stage and disappeared by the L4 stage (Table 3). Therefore, this 1.3-kb fragment promotes expression in P6.p at the time of *lin-39* upregulation, suggesting it may contain *cis*-regulatory sites for regulation by the Ras pathway.

To identify smaller elements responsible for specific expression, we made deletions of pJW5 (Fig. 4A). Surprisingly, we were unable to find a smaller fragment that was sufficient for expression in P6.p. Deleting as little as 120 bp (pJW5.3) or 300 bp (pJW5.4) from the ends of the 1.3-kb fragment abrogated GFP expression in P6.p. This suggests that P6.p expression may require multiple *cis*-elements spread throughout the 1.3-kb element, such that deletion of any of the sites drastically reduces expression (see Natarajan et al., 2004). The smaller constructs still expressed in the SM lineage (Fig. 4A). A 0.9-kb fragment (pJW5.5) was the smallest fragment sufficient for SM expression. We did not further delineate sequences mediating expression in the SM lineage.

Two conserved sequences necessary for lin-39 expression during vulval induction

We also took a phylogenetic approach to identify *cis*-elements responsible for expression in P6.p. A similarly located 1.3-kb region from *C. briggsae lin-39* drove GFP expression in the P6.p and SM lineages, indicating functional conservation (pJW5.Cb1; Fig. 4A and data not shown).

Table 3
Expression of pJW5 in the P6.p and SM lineages during development

Stage [a]	GFP expression P6.p lineage (%) [b]	GFP expression SM lineage (%) [c]
Early L2 (14 h)	0	0
Mid L2 (17 h)	3	57
Late L2 (19 h)	20	80
Early L3 (22 h)	40	97
Mid L3 (24 h)	57	93
Late L3 (26 h)	47	97
Early L4 (28 h)	3	93

[a] Larval stage and hour post-feeding at 25°C.
[b] Percentage of animals showing GFP expression in P6.p or its descendants.
[c] Percentage of animals showing GFP expression in the SM lineage. $n = 30$ for all time points.

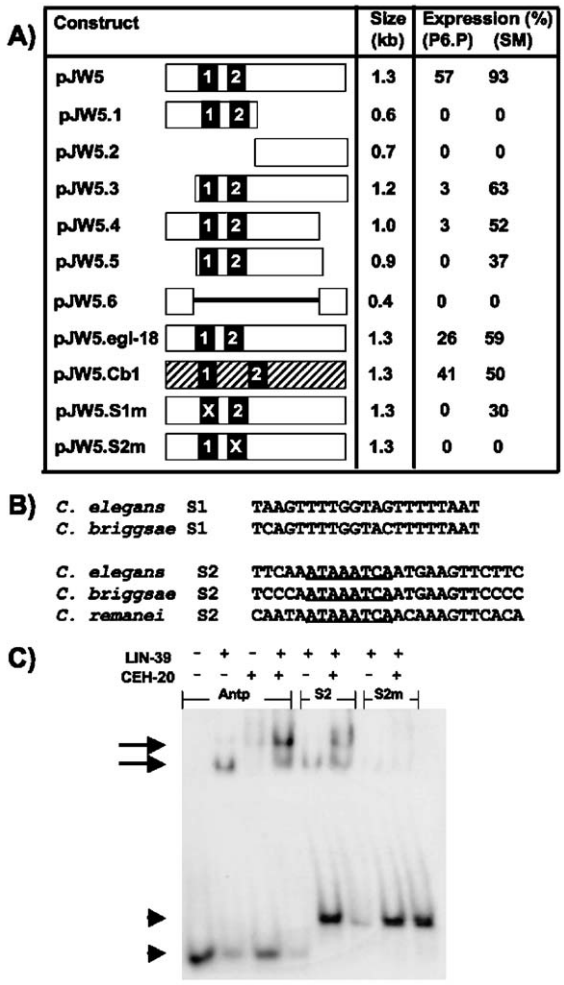


Fig. 4. Deletion analysis of pJW5 and binding of LIN-39/CEH-20 to site S2. (A) Indicated fragments were fused to the *pes-10::GFP* reporter (except for pJW5.egl-18) and scored as extrachromosomal arrays at 25°C during the mid-L3 stage (24 h post-feeding). Two or more lines per construct were scored with $n > 30$. Expression indicates percentage of animals with any expression in P6.p or SM lineages. Dark boxes represent sequences conserved between *C. elegans* and *C. briggsae*. An 'X' indicates mutation of the sequence. pJW5.Cb1 is the corresponding fragment from *C. briggsae*. pJW5.egl-18 indicates the 1.3-kb element was placed upstream of the minimal *egl-18::GFP* reporter. (B) Alignment of pJW5 sequences conserved between *C. elegans*, *C. briggsae* and *C. remanei*. Underline indicates the S2 Hox/Pbx binding site. (C) EMSA with His-tagged LIN-39 and CEH-20 proteins. Lanes 1–4: control site from the *Drosophila antennapedia* gene (Koh et al., 2002). Lanes 5, 6: wild-type site S2. Lanes 7, 8: site S2 with Hox and Pbx half-sites mutated. Arrowheads indicates free probes, arrows indicates protein:DNA complexes.

Comparing the sequence of these two fragments identified two conserved sequences, S1 and S2 (Fig. 4B; a third conserved sequence, S17, is described below). The S2 site is also conserved in the *lin-39* promoter sequence from *C. remanei* (Fig. 4B). Mutation of S1 caused a loss of GFP expression in P6.p and a reduction in the percentage of animals expressing GFP in the SM lineage (pJW5.S1m, Fig. 4A), while mutation of S2 caused a complete loss of GFP expression in both tissues (pJW5.S2m, Fig. 4A). Together with the deletion data, this indicates that multiple *cis*-acting regions in a 1.3-kb *lin-39* promoter fragment, including two small, evolutionarily conserved sequences, are necessary for expression in P6.p at the time of vulval induction.

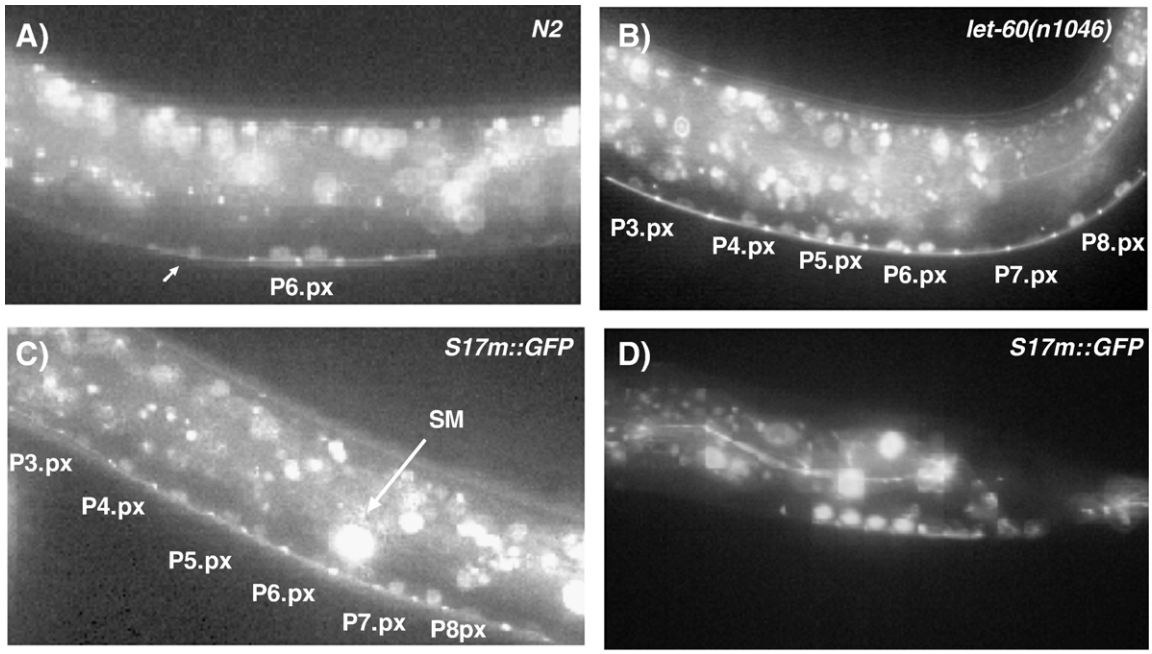
Conserved site S2 in pJW5 contains a LIN-39 binding site

A sequence within S2, TGATTTATTT, is similar to the Hox/Pbx heterodimer consensus site (TGATNNAT(G/T)(G/A) (Mann and Affolter, 1998) (Fig. 4B). Hox proteins can autoregulate their own expression in other species, and previous genetic data suggested that LIN-39 might autoregulate its expression in P6.p (Maloof and Kenyon, 1998). We found that purified LIN-39 bound to the S2 site alone in vitro, and bound in combination with CEH-20 (Fig. 4C). Mutation of the Hox and Pbx half sites (TCGCTTGCTT) caused a drastic decrease in binding of both LIN-39 and LIN-39/CEH-20 (Fig. 4C). This result demonstrates that LIN-39 binds in vitro to a sequence required for expression in the P6.p and SM lineages, indicating that LIN-39 may autoregulate its own expression in these cell types.

The activity of the 1.3-kb fragment is regulated by Ras signaling and lin-15

To test if pJW5 responds to Ras signaling in vivo, we used an integrated version of pJW5, *deIs6*, which showed P6.px GFP expression in 60% of animals (Figs. 5A, E). We introduced *deIs6* into *let-60(n1046)* mutants, in which the Ras pathway is activated in all the VPCs, causing a strong Multivulva phenotype (Beitel et al., 1990). In the activated Ras background, GFP expression was seen in all six VPCs (Fig. 5B), and the percentage of animals expressing GFP in P6.p showed a significant increase (Fig. 5E). We also crossed *deIs6* into *lin-45(n2018)* mutants which have a reduction-of-function mutation affecting the *C. elegans* Raf homolog. Ras signaling is reduced in these animals, leading to fewer than three VPCs adopting induced fates (Han et al., 1993; Hsu et al., 2002). In *lin-45(n2018)*; *deIs6* animals, GFP was expressed in P6.px cells in 20% of animals ($n = 30$), a significant decrease (Fig. 5E). These results show that the 1.3-kb fragment of the *lin-39* promoter is responsive to Ras signaling.

The SynMuv class of genes act redundantly to repress vulval induction, and encode proteins that control histone modification, chromatin remodeling and transcriptional repression, and other novel nuclear proteins (Ferguson and Horvitz, 1989; Fay and Han, 2000; Ceol and Horvitz, 2004). SynMuv genes were previously shown to alter *lin-39* expression (Chen and Han, 2001a,b). The *lin-15(n765)* mutation affects two SynMuv activities at the *lin-15* locus, resulting in a Muv phenotype (Huang et al., 1994). We found previously that a full-length transcriptional *lin-39::GFP* reporter was derepressed in a *lin-15 (n765)* background (Wagmaister et al., 2006). When we crossed *deIs6* into the *lin-15(n765)* background, GFP expression was seen in all six VPCs, similar to the result seen with *deIs6* in an activated Ras mutant strain (Fig. 5E). This indicates that the LIN-15 SynMuv gene products act directly or indirectly on the



E)

Strain	P3.px (%)	P4.px (%)	P5.px (%)	P6.px (%)	P7.px (%)	P8.px (%)
deIs6	0	0	0	60	0	0
let-60(n1046);deIs6	21	27	38	86*	27	38
lin-45(n2018);deIs6	0	0	0	20**	0	0
lin-15(n765ts);deIs6	57	43	30	53	13	27
lin-1(e1777);deIs6	3	10	10	7**	10	3
lin-31(n1053); pJW5	0	0	0	3	0	0
lin-31(+); pJW5[a]	0	0	0	47**	0	0
S17m::GFP	10	37	83	57	93	33
lin-31(n1053); S17m::GFP	3	10*	23**	27**	27**	7*

Fig. 5. pJW5 is responsive to Ras signaling, LIN-1 and LIN-31. GFP expression from (A) *deIs6* in a wild-type background; expression in two P6.p daughters (P6.px); (B) *deIs6* in *let-60(n1046)*; expression expanded to other VPC daughters (Pn.px); (C) *S17m::GFP* in wild type; expression expanded to other VPC daughters (Pn.px); (D) *S17m::GFP* in a wild-type male, showing expression in cells of the preanal equivalence group. All animals are mid-L3 larvae, with anterior to the left and dorsal to the top. Ventral GFP fluorescence from *ajm-1::GFP* highlights junctions of Pn.px cells; puncta indicate where cells abut (short arrow in (A)). Dorsal fluorescence is gut autofluorescence, or expression in intestinal nuclei (D). (E) Percentage of animals with expression in indicated VPC daughters (29–31 h post-feeding 20°C). $n > 30$ for all experiments. [a]*lin-31(+)* strain derived from *lin-31(n1053)*; *pJW5* by backcross. *indicates $P < 0.05$; ** indicates $P < 0.01$.

1.3-kb promoter fragment to repress *lin-39* expression in the VPCs.

The 1.3-kb fragment is regulated by lin-1 in vivo and bound by LIN-1 in vitro

LIN-1 is an ETS transcription factor that act downstream of the Ras pathway in the VPCs (Beitel et al., 1995). When *deIs6* was introduced into a *lin-1(e1777)* loss-of-function background, in which all the VPCs adopt vulval fates (Ferguson et al., 1987), the GFP expression domain was expanded to all six VPCs (Fig. 5E). This result is consistent with previous results showing that LIN-1 acts as a negative regulator of *lin-39* (Maloof and Kenyon, 1998; Wagmaister et al., 2006). However, we also found that GFP expression in P6.px was significantly reduced compared to *deIs6* (7% vs. 60%; $P < 0.01$; Fig. 5E). This suggests that LIN-1 also functions positively on *lin-39* expression in P6.p via the 1.3-kb fragment. These results suggest *lin-39* could be a direct target of LIN-1 in the VPCs.

ETS transcription factors bind to the core sequence GGA with a preference for the consensus sequence ACCGGA(A/T)(G/A)(C/T) (Nye et al., 1992; Shore et al., 1995; Sharrocks, 2001). LIN-1 binds to this consensus sequence in vitro (Miley et al., 2004); however to date, no LIN-1 binding site has been identified in a *C. elegans* gene. There are 34 putative ETS binding sites (EBS) containing the GGA core sequence in the

1.3-kb *lin-39* fragment (Fig. 6A; data not shown). We performed electrophoretic mobility shift assays with purified GST:LIN-1(1–278), which contains the ETS DNA-binding domain (Miley et al., 2004), and fragments of the 1.3-kb pJW5 element (Figs. 6A, B). We found binding of GST:LIN-1(1–278) to three of five fragments of pJW5, and to four of six subfragments containing 3, 9, 7, and 2 GGA sites respectively (Figs. 6A, B, and data not shown). Therefore, LIN-1 can bind in vitro to multiple sites within the pJW5 element.

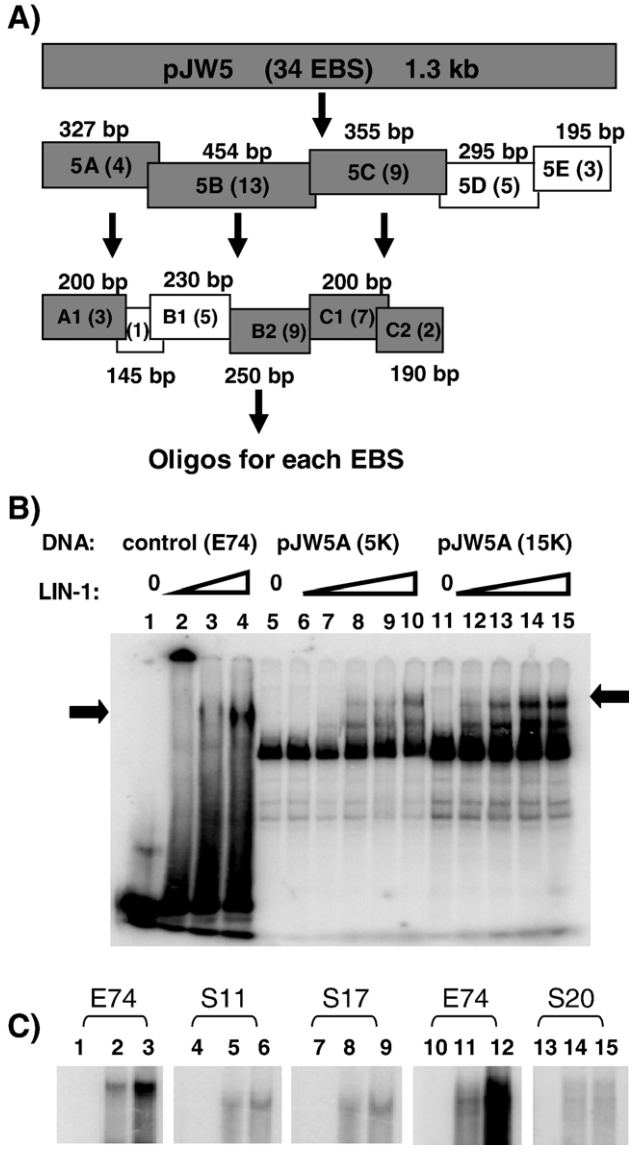


Fig. 6. LIN-1 binds multiple sites in pJW5. (A) Fragments of pJW5 used in EMSA experiments; number of putative ETS binding sites (EBS) is in parenthesis, size of fragment is shown. Fragments showing >10% of the binding seen with GST:LIN-1 and the E74 control site are shown in grey. (B) Representative EMSA with increasing amounts of GST:LIN-1(1–278). Lanes 1, 5, 6, 11—no protein; lanes 7, 12—20 ng; lanes 2, 8, 13—50 ng, lanes 3, 9, 14—100 ng; lanes 4, 10, 15—200 ng. Labeled probes were E74 control DNA (30,000 cpm; lanes 1–4); or JW5A fragment (5000 cpm; lanes 5–10 or 15,000 cpm; lanes 11–15). Arrow indicates complexes of GST:LIN-1 and DNA. (C) Binding of GST:LIN-1(1–278) to E74 (control), S11, S17 and S20 oligonucleotides. Lanes 1, 4, 7, 10 and 13—no protein; all other lanes—100 ng GST:LIN-1(1–278). Lanes 3, 6, 9, 12 and 15 have 60,000 cpm of labeled oligonucleotide; all others have 30,000 cpm labeled oligonucleotide. S11 and S17 are located in fragments 5B and 5B2, S20 is located in fragments 5C, 5C1 and 5C2.

We then tested binding of GST:LIN-1(1–278) to 17 oligonucleotides containing 21 GGA sites (Supplemental Table 1). LIN-1 was able to bind to three of these oligonucleotides (Fig. 6C): S11, containing the sequence AAACGGAAAGA (7% of E74 control binding); S17, containing the sequence GACGGAAGTT (16% of E74 control binding); and S20 containing the sequence AAGAGGAAGAC (2% of E74 control binding). Therefore, LIN-1 can bind in vitro to at least three single sites from the pJW5 *lin-39* promoter fragment.

LIN-1 represses expression in the VPCs via the S17 site

The S17 site showed the strongest LIN-1 binding in vitro and is the only one of the three sites that is obviously conserved upstream of the *lin-39* gene in all three *Caenorhabditis* species. The sequence TGACCAACTTCCGTC is found at −5.8 kb in *C. elegans*, −6.7 kb in *C. briggsae*, and −7.1 kb in *C. remanei*, and within 70 bp downstream of the conserved S2 site in all three species (Supplemental Fig. 2 and data not shown). We tested the role of S17 in vivo by mutating it in the context of the intact pJW5 GFP reporter, to create *S17m::GFP*. In *S17m::GFP* transgenic animals, GFP expression was present in all six VPCs, as seen with pJW5 in *lin-1(lof)* and *let-60(gof)* animals (Figs. 5C, E). In addition to being expanded to other VPCs, the GFP expression from *S17m::GFP* was also brighter than from pJW5 (data not shown). Therefore, conserved LIN-1-binding site S17 mediates repression of *lin-39* in VPCs where the Ras pathway is inactive. Unlike expression of *deIs6* in *lin-1(lof)* mutants, GFP expression in P6.p was not reduced in *S17m::GFP* animals (Fig. 5E, compare lines 5 and 8). This suggests that S17 is either not required positively for *lin-39* expression or is redundant with other positive elements. We also noted strong expression from *S17m::GFP* in cells of the preanal equivalence group in the male tail (Fig. 5D), another tissue where Ras signaling, Wnt signaling and Hox gene activity overlap (Emmons and Sternberg, 1997). In these cells, the domain of *S17m::GFP* expression was also expanded from that of *deIs6* (data not shown). In summary, we have identified a single site bound by LIN-1 in vitro that is necessary for repression in VPCs in which the Ras pathway is not activated. These results identify *lin-39* as a direct target of LIN-1-mediated repression in the VPCs.

The 1.3-kb fragment is regulated by lin-31 in vivo and bound by LIN-31 in vitro

LIN-31 is a Forkhead/winged-helix transcription factors that also acts downstream of the Ras pathway in the VPCs (Miller et al., 1993). LIN-31 has been proposed to act positively and negatively on vulval gene expression (Miller et al., 1993; Tan et al., 1998); however, no LIN-31 targets have been identified. We were unable to create a strain carrying *deIs6* in a *lin-31(n1053)* loss-of-function mutant background, so we generated a pJW5

extrachromosomal array in a *lin-31(n1053)* strain. In three *lin-31(n1053); Ex[pJW5]* lines, GFP expression was nearly absent in P6.px, with an average of 3% (n = 90) of animals showing expression (Fig. 5E). When we backcrossed the *lin-31(n1053); Ex[pJW5]* animals with wild-type animals, GFP expression was now seen in P6.px in 47% (n = 30) of the *lin-31(+); Ex[pJW5]* animals (Fig. 5E), indicating that expression in P6.p is dependent on *lin-31*. We also crossed *S17m::GFP* into a *lin-31(n1053)* background and found that expression was still seen in all six VPCs, but the level of expression in the population was reduced (Fig. 5E), and the intensity in each VPC was lower (data not shown). Therefore LIN-31 is still positively required for full expression when the S17 LIN-1 binding site is mutated. Based on these results, we tested if *lin-39* is also a direct target of LIN-31.

LIN-31 exhibits sequence-specific DNA binding in vitro to a site from the mammalian transthyretin (TTR) promoter that is bound by the Forkhead/winged-helix factor HNF-3γ; this binding is abolished by mutating the DNA site or by altering an amino acid in the DNA recognition helix of LIN-

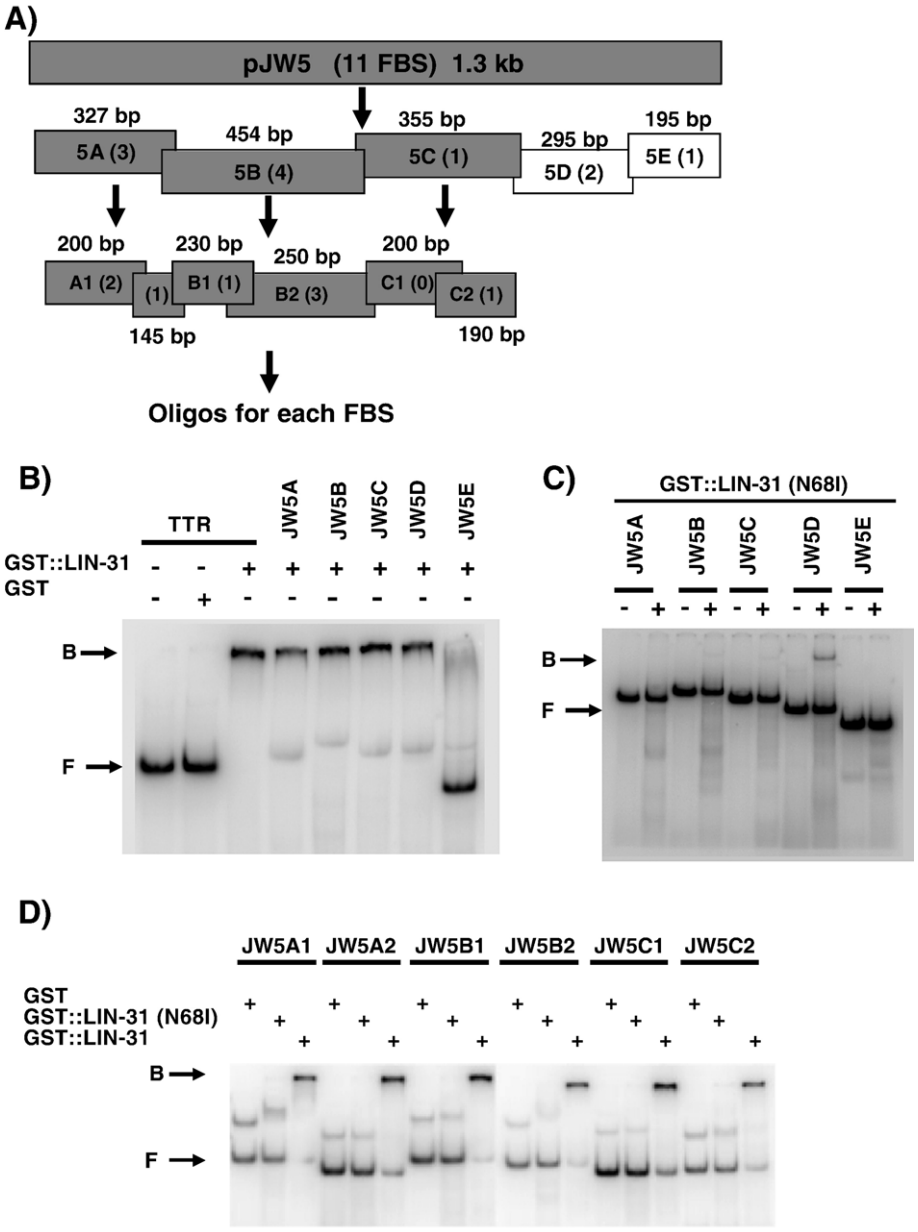


Fig. 7. LIN-31 binds multiple sites in pJW5. (A) Fragments of pJW5 used in EMSA experiments; number of putative forkhead binding sites (FBS) is in parenthesis, size of fragment is shown. Fragments bound specifically by LIN-31 are in grey. (B) EMSA using wild-type GST:LIN-31 protein and either control transthyretin promoter oligonucleotide (TTR, lanes 1–3) or pJW5 fragments 5A–5E (lanes 4–8). (C) EMSA using GST:LIN-31(N68I) carrying a point mutation in the LIN-31 DNA-binding domain and pJW5 fragments 5A–5E. Nonspecific binding to fragment 5D is seen. (D) EMSA using wild-type and GST:LIN-31(N68I) proteins with pJW5 subfragments A1, A2, B1, B2, C1, and C2. 'F' indicates free probe. 'B' indicates complexes with GST: :LIN-31. In panels (B) and (D), binding by purified GST alone is shown as a negative control.

31 (C. Morris and L. Miller, unpublished results). Using sites bound by other Forkhead/winged-helix transcription factors as a guide (Kaufmann and Knochel, 1996), we identified 11 possible Forkhead binding sites (FBS) in pJW5 (Fig. 7A and data not shown). Following the strategy outlined above for LIN-1, we found that purified GST:LIN-31 protein bound in vitro to six overlapping subfragments from pJW5, indicating that LIN-31 can bind multiple sites in the *lin-39* promoter fragment (Figs. 7A–D). However, unlike LIN-1, GST:LIN-31 did not bind strongly to any of several individual putative Forkhead binding sites we tested (data not shown), although the protein did bind well to a TTR site control oligonucleotide (Fig. 7B). This result suggests that either the LIN-31 binding site may not resemble those used by other Forkhead/winged-helix transcription factors, or that robust LIN-31 binding in vitro requires multiple LIN-31 binding sites and cooperative protein interactions. Further experiments will be needed to identify the precise LIN-31 binding sites in pJW5.

In summary, we showed that LIN-31 bound to several fragments from the 1.3-kb Ras-responsive element in vitro, and that loss of LIN-31 reduces expression from this element in vivo. Together these results suggest that LIN-31 likely acts as a direct positive regulator of *lin-39* expression in P6.p.

Discussion

The expression of developmental control genes such as Hox genes is regulated during development by transcription factors responding to internal and external cues. This regulation is mediated by *cis*-regulatory modules; complex DNA elements that can be over 1 kb in size, contain binding sites for multiple proteins, and which can function at a distance from the start of transcription (Davidson, 2001; Ochoa-Espinosa and Small, 2006). In this work, we isolated three *cis*-regulatory elements from the *C. elegans lin-39* gene that together account for a large part of the *lin-39* expression pattern. Further, we identified three *trans*-acting factors, LIN-1, LIN-31 and LIN-39, that bind to and regulate expression via one of these elements. *lin-39* is the first direct target of the Ras pathway in the VPCs identified to date.

cis-acting elements from the lin-39 gene

In our analysis of the *lin-39* genomic region, we characterized (1) a 338-bp fragment located 7 kb upstream of the *lin-39* ATG that directs expression in the P cells in the embryo and their progeny in the larva, (2) a 1.3-kb fragment located 5 kb upstream that is sufficient for expression in P6.p at the time of vulval cell fate specification, and for expression in the SM lineage, and (3) a 247-bp element from *lin-39* intron 1 that directs expression in VCNs (Fig. 8A).

Due to the large size of the *lin*-39 genomic region (over 24 kb), we chose to identify regions sufficient for expression when placed upstream of a minimal GFP reporter, rather than identify sequences necessary for expression by unidirectional deletion of a full-length *lin-39* reporter. While this strategy was successful, it is likely that we missed other important *cis*-acting

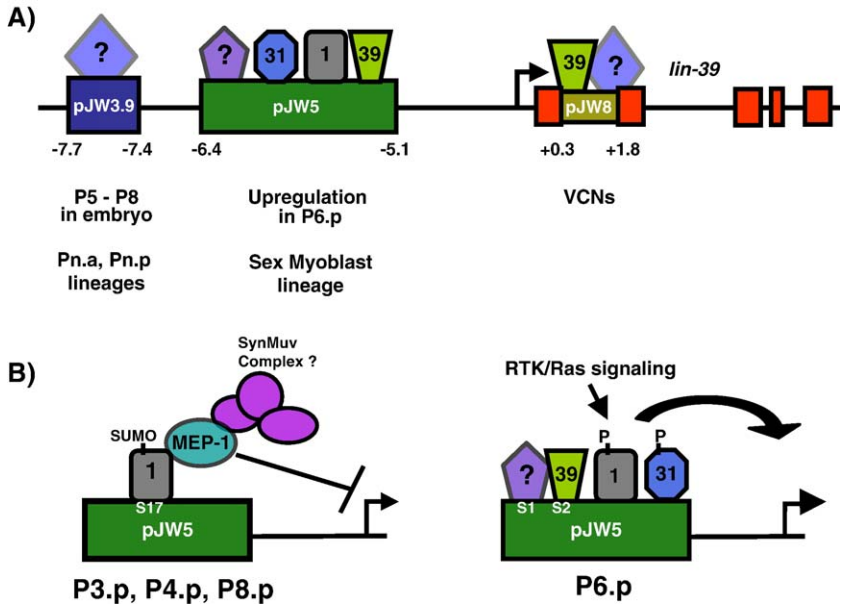


Fig. 8. *cis*-Acting sites and *trans*-acting factors regulating *lin-39* expression. (A) Red boxes indicate *lin-39* exons. The exact *lin-39* transcription start site (arrow) is not known. Three *lin-39* regions characterized in this work are indicated; below each is given their position relative to the ATG, and the sites of expression that they regulate. *trans*-Acting factors acting at each element are shown above the line; '31', '1' and '39' represent LIN-31, LIN-1 and LIN-39 respectively. Unknown factors are indicated by '?'. Other factors known to regulate *lin-39* expression, such as SEM-4 and LIN-25, are not shown. (B) Model for the regulation of *lin-39* expression via the pJW5 element at the time of inductive signaling. In VPCs in which the Ras pathway is not fully activated (P3.p, P4.p, P8.p), sumoylated LIN-1 binds to the S17 site to repress *lin-39* transcription. SynMuv gene products may be involved in this repression, directly or indirectly. In P6.p, activation of the Ras pathway leads to phosphorylation of LIN-31 and LIN-1, which, along with LIN-39, act positively to regulate *lin-39* expression.

sites. For example, we did not identify a *lin-39* Wnt-responsive element. There are numerous (>20) putative TCF binding sites located throughout the *lin-39* genomic region, so perhaps the Wnt-responsive sites are dispersed and no single fragment we analyzed contained a critical number of them, or they may require other noncontiguous enhancer elements for strong expression. Also, the proximal promoter region (2.5 kb), second intron, and 3′ UTR of *lin-39* all contain multiple sequences strongly conserved in both the *C. briggsae* and *C. remanei lin-39* genes (Supplemental Fig. 2), yet these elements did not drive significant GFP expression in our assay.

Identification of an element regulating lin-39 expression in the embryo and early larva

While several transcription factors and signaling pathways have been shown to regulate the expression of *lin-39* and other Hox genes during larval development, little is known about the initiation of Hox gene expression in *C. elegans*. *lin-39* expression is detected in embryos in the P cells P3-P8, and expression persists in the P cell progeny, including the VPCs (Wang et al., 1993; Maloof and Kenyon, 1998; Wagmaister et al., 2006). We isolated a 338-bp *lin-39* promoter fragment that directs GFP expression in the same pattern in the P cell lineage and identified three sequence elements within this fragment that are necessary for robust expression. The expression from pJW3.9 in the VPCs fades around the time of inductive signaling, suggesting that the role of this element is to mediate the early function of *lin-39* in the generation of the VPCs and to maintain *lin-39* expression until the time of Ras pathway activation. The identification of sequences controlling embryonic expression of *lin-39* is exciting, as they can be used to identify *trans*-acting factors that initiate *lin-39* expression.

LIN-1, LIN-31 and LIN-39 regulate lin-39 expression during vulval induction

At the time of vulval induction in the L3, *lin-39* expression increases in P6.p in a Ras signaling dependent manner (Maloof and Kenyon, 1998; Wagmaister et al., 2006). The pJW5 fragment of the *lin-39* promoter is sufficient for expression in P6.p and its descendants and is responsive to Ras signaling. The transcription factors LIN-1, LIN-31 and LIN-39 act downstream of Ras signaling in the VPCs, and we found that all three factors bind directly to pJW5.

To date, no direct targets of LIN-1 or LIN-31 have been identified. Only two direct targets of LIN-39 have been found, *hlh-8* (Liu and Fire, 2000) and *egl-18* (Koh et al., 2002), although *egl-17* is a likely candidate (Cui and Han, 2003). Ideally, to demonstrate that a particular gene is a direct target of a transcription factor, it is necessary to show that (1) the gene and the transcription factor have overlapping expression domains, (2) expression from the gene (or a reporter construct) is altered when the factor is absent or overexpressed, (3) the transcription factor binds to a site or sites in the gene, (4) disruption of this binding leads to misexpression of the gene, and (5) the factor is present at these sites in vivo. In the case of LIN-1, all of these criteria have been met. We and others have reported misexpression of *lin-39* in the VPCs in *lin-1* mutants (Maloof and Kenyon, 1998; Wagmaister et al., 2006). Here, LIN-1 was shown to bind multiple sites in the *lin-39* promoter, and mutation of one of these sites alters reporter gene expression in vivo. Finally, LIN-1 has recently been shown to be present at the *lin-39* promoter in vivo (F. Guerry and F. Mueller, personal communication). Therefore, *lin-39* is likely a direct target of LIN-1 in the VPCs.

Previous work showed that *lin-1* acts to inhibit induced vulval cell fates (Ferguson et al., 1987). It was proposed that in the absence of Ras signaling, sumoylated LIN-1 recruits chromatin remodeling factors to repress expression of genes required for vulval induction (Leight et al., 2005), and that phosphorylation of LIN-1 in response to Ras signaling may convert LIN-1 from a repressor to an activator of transcription (Howard and Sundaram, 2002; Miley et al., 2004). Consistent with this, our data show that LIN-1 binds to the pJW5 element and represses expression in cells in which the Ras pathway is inactive, and that ectopic activation of the Ras pathway overcomes this repression. This repression depends on the S17 LIN-1 binding site (Fig. 8B). Repression may be mediated by SynMuv gene products, since ectopic expression is observed when *lin-15* is mutated, although it is not known whether this is a direct or indirect effect on the *lin-39* promoter (Fig. 8B). However, we also found that in *lin-1* mutants, expression from pJW5 in P6.p was significantly decreased, suggesting that LIN-1 also acts positively on *lin-39* expression. Indeed, genetic data suggest that *lin-1* is required positively for expression of several genes (Howard and Sundaram, 2002; Tiensuu et al., 2005). Therefore, we propose that LIN-1 acts both negatively and positively on the same gene, *lin-39*, in a manner dependent on Ras pathway activation in the VPCs (Fig. 8B).

lin-31 encodes a member of the winged-helix family of transcription factors acting downstream of Ras signaling in the VPCs (Miller et al., 1993), and LIN-31 is a substrate for MAP kinase in vitro (Tan et al., 1998). The phenotype of *lin-31* null mutants suggests that *lin-31* is required to both promote and repress induced vulval fates, and the DNA-binding domain of LIN-31 is required for these functions (Miller et al., 1993, 2000). Based on these data, it was proposed that LIN-1 and LIN-31 physically interact to repress target gene expression, and that phosphorylation of both factors disrupts this interaction, allowing LIN-31 to activate transcription (Tan et al., 1998). We found that LIN-31 binds to multiple fragments from pJW5 in vitro, and that expression from pJW5 and S17m::GFP is reduced in a *lin-31* mutant background. Further, loss of *lin-31* did not result in derepression in other VPCs, as in a *lin-1* mutant. These results are not consistent with the model that LIN-31 acts to repress transcription in the absence of Ras signaling but indicate that *lin-31* is positively required for *lin-39* expression and suggest that *lin-39* may be a direct target of LIN-31 in P6.p (Fig. 8B). Curiously, upregulation of a *lin-39* transcriptional reporter containing the entire *lin-39* genomic region was not dependent on *lin-31* (Wagmaister et al., 2006). Rather than invalidating the model that *lin-39* is positively regulated by LIN-31 in P6.p, we believe this suggests that

redundant mechanisms operate in the intact promoter to ensure P6.p expression.

Finally, we found that LIN-39 itself bound to a sequence in pJW5, and that mutation of this sequence abolished expression in P6.p and the SMs. We did not examine whether expression from pJW5 is dependent on *lin-39* activity, since *lin-39* null mutants lack VPCs at the time of expression from pJW5 (Clark et al., 1993; Wang et al., 1993). Maloof and Kenyon showed that LIN-39 upregulation in P6.p is defective in a *lin-39* mutant that produces a protein with a substitution in the DNA-binding domain, suggesting that *lin-39* upregulation requires LIN-39 activity (Maloof and Kenyon, 1998). The demonstration that LIN-39 binds to several sites in the *lin-39* gene, combined with these previous data, strongly suggests that LIN-39 autoregulates its own expression (Fig. 8B). Consistent with this, LIN-39 can activate transcription in yeast when brought to DNA by the GAL4 DNA-binding domain (J. A. Wagmaister and D. M. Eisenmann, unpublished results). Autoregulation provides a mechanism by which the pattern of *lin-39* expression initiated early in development can be maintained at later stages when the initiating cues or factors may no longer present. Alternatively, another homeodomain protein could bind to this site in vivo to positively regulate *lin-39*. In the future, chromatin immunoprecipitation assays will allow us to determine if LIN-39 is bound to this site in vivo.

In summary, we have characterized two *cis*-regulatory modules from *lin-39* that regulate embryonic and larval expression in the P lineage, and Ras-dependent upregulation in P6.p. In addition, we have shown that *lin-39* is likely to be a direct target of LIN-1 and LIN-31, and to autoregulate its own expression. Future work will concentrate on showing that these transcription factors are bound to the *lin-39* promoter in vivo, and determining the identity of other *trans*-acting factors required for the initiation and regulation of *lin-39* expression in distinct cells during development. In this way, we hope to expand out knowledge of the initiation, maintenance and regulation of expression of Hox genes in nematodes.

Acknowledgments

We thank Jun Kelly Liu and Andy Fire for LIN-39 and CEH-20 expression plasmids, *pes-10*::*GFP* reporter plasmids and the *pha-1* strain, Xiang Li and Chuck Bieberich for assistance in protein purification, and Brandon Le for assistance with statistical analysis. We thank Jessica Siegel for technical assistance. We thank Frédéric Guerry and Fritz Müller for sharing unpublished results. We thank Pradeep Joshi and other members of the Eisenmann laboratory for insightful discussions and critical reading of the manuscript. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources (NCRR). This work was supported by NIH grant GM65424 and NSF grant IBN-0131485 to DME. L. M. was supported by NSF grant IBN-0315072. C.M. was also supported by Howard Hughes Medical Institute grant 71100562401 to Santa Clara University. K.K. is a Scholar of the Leukemia and Lymphoma Society and was supported by the NIH.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.05.008.

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